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CONTRACTING ORGANIZATION: The Jackson Laboratory
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13. ABSTRACT *(Maximum 200*

During the first year of this grant-in-aid, we have expanded the C57BL/6J, C3H/HeJ, and B6C3F1 mouse colonies to provide mice for biochemical and histomorphometric analyses at ages from 8 to 30 weeks. Serum bone formation marker data gathered include osteocalcin and alkalin phosphatase. Our best serum bone resorption marker was osteoclastic acid phosphatase. In addition, we have measured serum Insulin-like Growth Factor-I, mouse Growth Hormone, and 24hr urinary creatinine. Morphologic data gathered on duel-labeled bone samples include cross-sectional areas, labeled surfaces, mineral apposition rates, and bone formation rates at periosteal and endosteal surfaces of tibias. These data primarily reflect changes in cortical bone compartment. Genetic differences discriminating C57BL/6J from C3H/HeJ mice as early as 8 weeks of age were found in nearly all of the measures taken. Differences in both bone formation and resorption underlie the differences in peak bone density that characterize these inbred strains, supporting our hypothesis of polygenic regulation of bone density. The acquisition of this data base will facilitate identification of the bone biologic parameters associated with peak bone density that are regulated by genes being mapped in the second specific aim of this project.

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FOREWORD

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____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Wesley D. Beamer 10/20/97

PI - Signature Date

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LOMA LINDA UNIVERSITY

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Department of Medicine

Loma Linda, California 92350
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LETTER OF INTENT TO ENTER INTO A CONSORTIUM AGREEMENT

TITLE OF APPLICATION: Genetic and Dynamic Analyses of Murine Peak Bone Density

APPLICANT ORGANIZATION: The Jackson Laboratories, Bar Harbor, ME

PRINCIPAL INVESTIGATOR: Wesley G. Beamer, Ph.D.

COOPERATING INSTITUTION: Loma Linda University, Loma Linda, CA

PROPOSED PROJECT DATE: September 27, 1997 through September 26, 1998

The appropriate program and administrative personnel of each institution involved in this grant application are aware of the PHS and National Science Foundation Consortium grant policies and are prepared to establish the necessary inter-institutional agreement consistent with carrying out this project.

Loma Linda University is in compliance with PHS and NSF policies regarding Animal Care and Use, Human Subjects, civil Rights, Handicapped Individuals, Sex Discrimination, Scientific Fraud (Misconduct) Assurance, Delinquent Federal Debt, Debarment and Suspension, Drug-Free Workplace and Lobbying.

Cooperating Institution

By: _____

Date: 9/29/97

David J. Baylink, M.D.
Principal Investigator, LLU
Typed Name and Title

By: _____

Date: 9/29/97

J. Patrick Yhip, C.P.A.
Director of Grants Management
Typed Name and Title

Applicant Institution

By: _____

Date: 10/24/97

Wesley G. Beamer, Ph.D.
Principal Investigator
Typed Name and Title

By: _____

Date: 10/24/97

Lucy L. Witt, Manager
Grants Management Services
Typed Name and Title

prepared 9/10/97
revised 9/10/97

Year 2 Budget for Army OP Grant (Based Award)
Budget period: 9/27/97 through 9/26/98

Personnel	Base	Time	Salary	33.50% Fringe	Totals
Postdoctoral Fellow	29,673	100%	29,673	9,941	39,614
Laboratory Tech.	22,849	50%	11,425	3,827	15,252
	Total Personnel		41,098	13,768	54,866
Equipment	Total Equipment				-
Supplies	Histology Supplies		7,928		
	Total Supplies				7,928
Travel	1 national meeting and 1 visit to Jackson Labs				3,423
Other Expense (publications $1,100; and office supplies $500)					1,680
			Subtotal Direct		67,897
			Overhead @ 26%		17,653
			Grant Total		85,550

	(Rebudgeted) Initial Period	**Year Two**	Year Three	Year Four	Year Five	Grand Total
Personnel	38,897	**54,866**	57,061	-	-	150,824
Equipment	17,145	**-**	-	-	-	17,145
Supplies	7,550	**7,928**	8,324	-	-	23,801
Travel	3,260	**3,423**	3,594	-	-	10,277
Other	1,600	**1,680**	1,764	-	-	5,044
Subtotal	68,452	**67,897**	70,743	-	-	207,091
Indirect	13,340	**17,653**	18,393	-	-	49,386
Total	81,792	**85,550**	89,136	-	-	256,477

Indirect Cost Rate: 26%
Based on LLU off-campus and MTDCB.

NOTE: Year 2 budget reflects a 5% inflation adjustment in all categories except personnel.
Personnel costs have been increased by 8%, inclusive of fringe benefit rate increase.
Fringe benefit rate has increased to 33.5% effective July 1, 1997.
Year 2 budget is based on the initial period approved budget.

Budget Approved:

David J. Baylink, M.D.
Principal Investigator

J. Patrick Yhip, C.P.A.
Director of Grants Management

INTRODUCTION

Osteoporosis and the importance of peak bone density

Osteoporosis is a disease of insufficient bone density. In the human skeleton, bone density increases with age, reaching peak levels at 18-22 years of age (1-3). As bone density decreases with age, the risk of osteoporotic fractures increases, especially when the density falls below the fracture threshold. This relationship suggests that the risk of osteoporotic fracture can be defined in terms of two characteristics - the peak bone mineral density (BMD) achieved and the net bone loss rate. The subject of this application is peak BMD, since theoretically, a person with a higher peak BMD would be less likely to fall below the fracture threshold than a person with a lower peak BMD, if both lost bone at a similar rate.

During the past several decades, considerable progress has been made in understanding the pathogenesis of osteoporosis with respect to cellular mechanisms that account for age-related bone loss, but we still know very little about the mechanisms that determine peak BMD. This gap in our understanding has become critical because of recent evidence that as much as 70% of the variation in peak BMD can be accounted for by genetic factors (4). To identify such heritable factors in humans, investigations have primarily focused on unrelated populations to find association of BMD and restriction fragment length polymorphisms (RFLPs) in genes thought to be involved with BMD. One such factor, the vitamin D3 receptor (VDR) has received immense attention with equal numbers of reports showing or not showing a significant relationship between BMD and VDR RFLPs. Other studies have looked for relationships between BMD and serum PTH, collagen, GH levels, and serum mineral. These population studies are subject to sampling bias, lack of knowledge about how many alleles ther are, lack of knowledge about parental sources of genetic alleles, and have low statistical resolving power.

Genetic analysis of peak bone density

Evidence for genetic regulation in humans. In contrast to population association studies, recent studies have disclosed less variation of BMD in young adult monozygotic twin pairs than in dizygotic twins (4-6). In a study of 71 juvenile and 80 adult male dizygotic and monozygotic twins, Smith et al. found significantly larger variation in radial bone mass and width in dizygotic than in monozygotic twins, indicating the importance of genetic regulation of bone mass (7). In a cross-sectional study of mother-daughter pairs, premenopausal bone density had a strong familial resemblance at the distal forearm, lumbar spine, and proximal femur (8). McKay et al. also reported a strong familial resemblance in lumbar and femoral BMD among mother-daughter and mother-grandmother pairs, although some discordance was noted between the hip and spine sites (9).

Kelly et al. reported a strong genetic effect on serum osteocalcin, a well recognized biochemical marker of bone formation, in both pre- and post-menopausal twins (10). More significantly, within-pair differences in osteocalcin in dizygotic twins predicted within pair differences in bone density at the lumbar spine and femoral neck, suggesting that genetic effects on bone formation relate to bone density. Furthermore, Morrison et al. reported that RFLPs in the VDR gene predicted the variance in serum osteocalcin, whereby both low serum osteocalcin values and the *bb* VDR genotype are associated with a high peak BMD (11).

Difficulties associated with human studies of peak bone density. Genomic diversity and generation time for the human population makes assessment of genes regulating bone density a very difficult undertaking. Twin studies and associative studies with unrelated individuals have substantiated estimates of heritability and provided ways to test relationships between DNA polymorphisms and osteoporosis related traits. However, each approach has its limitations, e.g. relative scarcity of twin pairs, inability to determine number of loci involved, inappropriate evidence to support selected candidate genes, cannot assess interaction between genes and

environment, etc. Accompanying the rich genomic heterogeneity of the human population is an equally varied environment. Although some aspects of environmental variability can be controlled for limited periods of time and others can be assessed by questionnaire, compliance and veracity are always problematic. Long-term studies required to identify critical environmental variables apart from genetic effects are simply not cost effective or practical. Therefore, there is a need for an animal model whose genome and environment can be experimentally manipulated to reveal genes and biochemical mechanisms for acquisition of peak bone density.

Mouse model for genetic analysis of BMD. We have adapted quantitative computerized tomography to develop a genetic model consisting of two inbred strains of mice, C57BL/6J (B6) and C3H/HeJ (C3H), with highly significant differences in peak BMD in vertebrae (12%), tibia (20%), and femur (53%), with which to identify genes responsible for peak BMD (12). Preliminary data showed that C3H mice (highest BMD) differed from B6 (lowest BMD) mice as follows: 1) reduced medullary cavity volume and increased cortical thickness; 2) increased metaphyseal trabecular BMD; 3) decreased serum osteocalcin, serum skeletal alkaline phosphatase, and urine crosslinks/creatinine; and 4) decreased osteoclast number at both endosteal cortex and trabecular bone and decreased osteoclast proliferation in marrow cultures at 2 months of age. These morphological and function differences between B6 and C3H mice are genetically founded and strongly suggest that differences in rates for bone formation and resorption account for differences in BMD.

Inbred strain mice, such as B6 and C3H, represent unlimited numbers of genetically identical "twins" whose genes can be experimentally analyzed and whose environments can be strictly controlled. Equally important, each inbred strain is genetically different from every other inbred strain, making possible planned matings and studies of segregating genes. Over the past 10 years, the mouse genome has become highly defined, especially with respect to protein and molecular polymorphic differences among the various inbred strains. This detailed marking of chromosomes greatly facilitates rapid location of new genes. In addition, segments of many chromosomes have been identified that share identical marker loci in mouse and man (13). These homologous segments have provided important clues to mapping human genes associated with a number of diseases including Waardenburg syndrome (14, 15) and retinitis pigmentosa (16). Substantial computerized genetic data bases maintained at The Jackson Laboratory are available to support mapping and biological research efforts that utilize the mouse genome to determine human genetic regulation.

The research proposed in this application focuses on genes that regulate BMD in phenotypically normal inbred strains of mice. Because inbred strains exhibit a normal phenotype and an unmanipulated genome, these models of bone regulatory genes are different from murine models that arise from a) spontaneous mutations (osteopetrosis, 17; hypophosphatemia, 18), b) induced mutations resulting from insertion of foreign genes (human *IL-4*; 19), targeted mutated genes (*Mov 13*; 20), or knocked-out genes (*src*-deficient mice; 21).

Quantitative trait loci mapping techniques. Quantitative traits are distinguished from traits with discrete classes (coat color, enzyme deficiency, protein structure variant, or DNA difference that could be detected by Southern blots or PCR) by having a distribution that is more like a bell shaped curve, wherein the extreme ends of the bell shaped curve may predispose to disease. Such traits are determined by many genes and are often strongly influenced by environment.

Quantitative traits are the basis for many common diseases: hypertension, atherosclerosis, obesity, type II diabetes, and osteoporosis. Until recently, mapping genes that underlie quantitative traits was not possible, but in the last few years three major advances have come together that now permit geneticists to map the genes underlying quantitative traits.

First, the idea of how to map quantitative trait loci (QTL) was developed by Lander and Botstein (22). The concept is to carry out a cross between two parents that differ in one or more quantitative traits and measure the backcross or F2 intercross progeny for the trait of interest. The extreme ends of the bell shapedphenotype distribution are used for genome analysis. This can be

done because the individuals at the extreme high end, for example, will carry all or most of the alleles that cause an individual to be high. Most investigators choose to genotype mice in the upper or lower 5 or 10% of the phenotype distribution. The entire genome is then scanned by testing for polymorphic markers at intervals of about 15-20 cM. The mouse genome has 1600 cM, so a minimum of 100 markers cover it reasonably well. If the allele that determines the high end of the quantitative trait is designated H and the one determining the low end is called L, then the simplest statistical analysis tests for an excess of H alleles among the individuals at the high end of the distribution and an excess of L alleles at the low end of the distribution.

Second, a major advance is the availability of a large number of molecular markers that are easy to use. During 1995 the Genome Center at MIT completed its task of developing 6000 polymorphic markers for the mouse (23). These are based on common genetic variations in the length of CA repeats found throughout the mouse genome. The primer pairs are inexpensive ($20/pair) and can be assayed by PCR.

Third, the development is that of computer programs and statistical methods to analyze the large amounts of data. This area is still rapidly evolving. Currently available computer programs are MapMaker (24), QTLCartographer (25-28), and the new MapManagerQT by Dr. Ken Manly of Roswell Park Memorial Institute, Buffalo, NY. All these programs are available at The Jackson Laboratory and our collaborator, Dr. Frankel, skilled in their use.

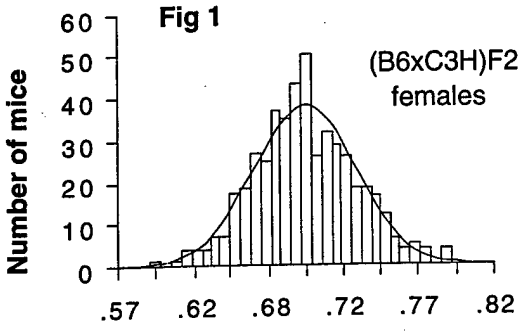
Our proposal represents the first attempt to use QTL mapping for the analysis of bone density in B6C3F2 mice. The major problem was not the genetic infrastructure, but the need for reliable methodology for measuring bone density that could be applied to large numbers of mice. This methodological problem has been solved with the peripheral quantitative computerized tomography (XCT 960M) described in Beamer et al. (12). Once major genes have been identified by the first QTL mapping cross (i.e., B6C3F2 mice), further statistical and genetic techniques are able to isolate and study the individual genes.

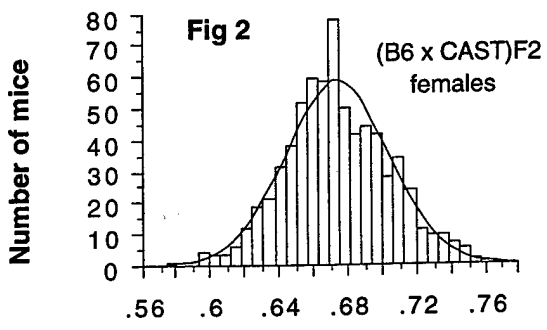
BODY

Progress on Specific Aim 1 (Conducted at The Jackson Laboratory, Bar Harbor, ME). To test the hypothesis that the major genes determining the interstrain difference in BMD can be mapped by Quantitative Trait Loci (QTL) analysis of intercross F2 progeny between C57BL/6J (B6) and C3H/HeJ (C3H) mice. Femoral volumetric BMD is assessed by pQCT using the XCT 960M. Genotyping is performed on kidney genomic DNA using PCR based SSLP that distinguish B6 from C3H alleles.

a) Provision of mice for research projects. All inbred and hybrid mice used in **Aims 1** and **2** are raised in Dr. Beamer's animal space at The Jackson Laboratory, Bar Harbor, ME. Colonies of B6, C3H, B6C3F1, and B6C3F2 mice are providing subjects for genetic and endocrinologic analyses carried out in Bar Harbor, ME, and biochemical and cell biology analyses carried out in Loma Linda, CA. This collaboration wil continue into all remaining years of the grant period. This collaboration maximizes the efficient use of mice and facilitates co-ordination and interpretation of research results.

b) Genetic analyses. Our preliminary data indicated that 60-70% of the variance in bone mineral density of B6 and C3H mice is genetically based. Genes responsible for the difference in peak bone density between C3H and B6 mice are being mapped via quantitative trait loci (QTL) analyses of F2 progeny from intercrosses of (C57BL/6J x C3H/HeJ)F1 parents. The goal is to produce 1000 females for genetic analyses. As of June 1997, 485 B6C3F2 females have been raised to 4 months of age, then necropsied to obtain serum, body weights, kidneys and skeletal specimens. Femoral bone density has been measured by pQCT (XCT 960M) and shown to be normally distributed curves for both total and cortical bone densities. Genomic DNA was prepared from kidneys of approximately 50 mice represented in each extreme tail of the cortical density distribution shown in **Figures 1** and **2** below.





Femoral cortical density (mg/mm3)

Genotyping of these 100 mice is still in progress, and, at this stage, is based solely on genotyping for simple sequence length polymorphisms (SSLPs) using PCR methodology that discriminates B6 from C3H alleles at each locus. A total of 100 loci have been typed in 48-81 F2 progeny for all 19 autosomes, with the objective of typing an average 5 loci/chromosome. Using Ken Manly's Mapmanager QT program for analysis of markers that are associated with high or low bone density, we have found two loci meeting Lander and Kruglyak (29) criteria for highly significant linkage with bone density (Chr 1, $p < 10\text{-}5$; Chr 4, $p < 10\text{-}9$) and four loci meeting criteria for suggestive linkage ($p < 0.001$; one on Chr 5 and 18; two on Chr 6). With respect to mode of inheritance, thus far C3H alleles at these loci have behaved as either dominant or additive, with C3H alleles having strong effects.

A parallel mapping study for genes underlying bone density has been carried out with B6CASTF2 progeny from intercrosses of (B6 x CAST)F1 parents. The objectives of this study were to gain converging lines of evidence regarding loci in B6 that regulate bone density. We have genotyped 689 F2 progeny for 121 loci on the 19 autosomes. The distribution of both femoral total and cortical bone densities are Gaussian in form, as are those for B6C3F2 progeny (graphed above). Mapmanager QT statistical analyses indicated that there are three loci significantly correlated with bone density ($p < 0.0001$; Chr 1, 3, 5) in B6 versus Castaneus, and up to five additional chromosomes with loci showing suggestive linkage ($p < 0.01$; Chr 10, 13, 14, 15, 18). As was true for the C3H alleles above, the CAST/EiJ alleles typically are additive and most contribute to a higher bone density.

c) Regulation of bone density in B6 and C3H mice. In preparation for developing biological interpretations of genetic mapping data for bone density, we have investigated the regulation of bone density by four recognized perturbing stimuli.

1) Ovariectomy (OvX). Effect of gonadal steroid deprivation was analyzed in retired breeder C3H and B6 females that had been held for an additional 6 wks post lactation. Groups of 8 mice were OvX or sham treated, then necropsied for bone and tissue samples at 2, 4, 6 and 8 wks post surgery. Results showed that OvX induced expected responses in both genotypes to (1) modestly increase body mass, and (2) to lose uterine mass. Femoral BMD declined in B6 mice after 2 wks and remained depressed through 8 wks, whereas BMD remained unaffected by OvX through 8 wks post surgery.

2) Unilateral neurectomy of rear limb to mechanically 'unload' tibiae. Young adult 4 month old B6 and C3H female mice were unilaterally neurectomized (sciatic, femoral, & obturator nerve section) to completely desensitize and eliminate all muscle tension on the left tibia. Groups were necropsied at 2 or 4 wks post surgery and the proximal 1\3 of tibias assessed

for changes in physical dimensions and density by pQCT (XCT 960M) at 1 mm intervals. Data for neurectomized tibiae were compared with both opposite limb and with sham neurectomized groups. We found that unilateral neurectomized B6 mice showed loss of periosteal circumference and BMD at 2 wks and 4 wks post surgery, whereas C3H mice did not lose bone size or BMD at either time post surgery. Remarkably, C3H bone does not appear to respond to mechanical stimuli.

3) Exogenous glucocorticoid. Groups of young 8 wk old B6 and C3H females were placed on control, low (10 μg/ml), or high (50 g/ml) dose prednisolone (Pediapred) treated water to determine if this agent with well documented bone resorptive promotion properties would exert negative regulation on bone growth and mineralization. Dose levels were selected to approximate those used in pediatric clinical practice. Mice were necropsied after 1, 2, or 6 wks of treatment. Glucocorticoid treatment for 1 or 2 wks resulted in marginal changes, whereas 6 wks high dose treatment effects were clear. Somatic growth (inc. body mass) was reduced in B6, whereas C3H body weight gain was unaffected. Both spleen and thymic weights (mg/g Bwt) were reduced in a dose dependent fashion in both B6 and C3H. Skeletal growth (femur: mm/g B wt) was reduced in B6, whereas C3H femurs were unaffected. Both total and cortical femoral density were reduced in a dose dependent fashion.

4) Serum IGF-I. Twelve separate RIA's of serum from B6 and C3H repeatedly confirmed that C3H IGF-I levels are significantly higher than B6 levels. Replicate RIA's of F2 mice drawn from the high or low bone density ends of the distribution (Fig 1) showed that high IGF-I levels segregated with high bone density and low IGF-I levels with bone density. These data suggest that IGF-I is one of the factors accounting for differences in bone density between B6 and C3H mice. Interestingly, none of the loci mapped in B6C3F2 mice thus far corresponds to candidate genes such as GH, Ghrh, Ghrhr, IGF-I, IGFr, or serum IGF Binding Proteins. Dr. Baylink in Loma Linda has confirmed earlier reports showing that serum GH is also higher in C3H compared with B6.

Progress on Specific Aim 2 (Conducted at Loma Linda University, Loma Linda, CA). To identify the bone modeling (i.e., bone formation and bone resorption) mechanisms that characterize the skeletal phenotypes of B6 and C3H mice.

To accomplish this, we will conduct longitudinal studies during development of peak bone density in B6, C3, and F1 and F2 hybrid mice at 4, 6, 8, 16, and 26 weeks of age for measurements of: a) biochemical assays of bone formation (serum osteocalcin and serum skeletal alkaline phosphatase) and bone resorption (serum osteoclastic acid phosphatase); and b) histomorphometric measurements of bone formation and resorption in trabecular bone of the femoral metaphysis and in periosteal and endosteal bone of the femoral diaphysis.

a) Biochemical Assays of Bone Formation (serum osteocalcin and serum skeletal alkaline phosphatase) and Bone Resorption (serum osteoclastic acid phosphatase).

1) Bone Formation Markers

(a) Osteocalcin is a bone specific protein (46 amino acids) secreted by osteoblasts. It comprises 1-2% of the total bone protein. A fraction of newly synthesized intact osteocalcin (about 15%) is released into the circulation, where its concentration reflects osteoblastic activity and bone formation. The radioimmunoassay (RIA) used for the measurement of osteocalcin in mouse serum, utilizes an antibody generated in goats against intact mouse osteocalcin. Purified intact osteocalcin from mouse bones is used as tracer and calibrator. The assay sensitivity defined at 95% confidence limits was 1.56 ng/ml. The intra-assay (n=10) and interassay (n=3) variation (CV) of the assay at low and high concentrations were between 5.61% and 14.9 %. Average recovery of osteocalcin upon dilution of mouse serum up to 40 fold was 110.4%.

Our data on serum osteocalcin are given in **Table 1** below.

Table 1. Serum osteocalcin in B6,C3H and B6C3F1 mice.

Age	C57BL/6J (B6) ng/ml Mean±SD (n)	C3H/HeJ (C3H) ng/ml Mean ± SD (n)	B6C3HF1 ng/ml Mean ± SD (n)	P
8 Week	401 ± 78.1 (20)	360 ± 61.21 (40)		<0.05
12 Week	248 ± 48.4 (15)	214 ± 49.6 (20)	208 ± 38.03 (29)*	<0.001
16 Week	253 ± 35.1 (15)	167 ± 35.4 (32)	256 ± 30.6 (32)**	<0.001
26 Week	171 ± 31.2 (20)	118.8 ± 23.5 (19)		<0.001
30 Week	(VIRG)238 ± 37.8 (13)	170 ± 22.3 (5)		<0.001

*Significantly different from 'B6' but not significantly different from 'C3H'
**Not significantly different from 'B6'

These data in **Table 1** show that the serum osteocalcin measure of bone formation is significantly greater at 8, 12, 16, 26 weeks and 7.5 months in the B6 than in the C3H animals. At 12 weeks (but not 16 weeks), the F1 values for serum osteocalcin were significantly different from the B6 but not from the C3H mice.

(b) Alkaline Phosphatase. Measurement of bone specific alkaline phosphatase (sALP) has been shown to provide an index of the rate of bone formation. Alkaline phosphatase can be measured in serum by kinetic methods using p-nitro phenylphosphate as substrate. In mice, the major isoform of alkaline phosphatase (ALP) that can interfere in the serum measurements of sALP is the intestinal alkaline phosphatase. However, activity of this intestinal isoenzyme is more sensitive to inhibition by L-phenylalanine. By the addition of 15mM L-phenylalanine, up to 90% of intestinal alkaline phosphatase can be inhibited without significantly affecting the skeletal isoenzyme activity. The phenylalanine inhibition assay exhibited intra-assay (n=10) and inter-assay (n=8) variation (CV) between 1.9% and 3.8%. The assay can detect <10 mU/ml of alkaline phosphatase in mouse serum.

Our data on serum skeletal alkaline phosphatase in the B6,C3H and B6C3HF1 mice are provided in **Table 2** below.

Table 2. Serum skeletal alkaline phosphatase levels in B6, C3H, and B6C3F1 mice.

Age	C57BL/6J(B6) mU/ml Meanl SD (n)	C3H/HeJ (C3H) mU/ml Meanl SD (n)	B6C3HF1 mU/ml Meanl SD (n)	P
8 Week	75.53 ± 10.9(20)	70.68 ± 8.32(40)		0.056
12 Week	48.06 ± 4.40 (15)	49.80 ± 4.33 (20)	47.5 ± 9.77 (30)	NS
16 Week	38.95 ± 7.78 (15)	41.26 ± 4.72 (32)	40.16 ± 5.12 (31)	NS
26 Week	34.52 ± 4.18 (29)	34.64 ± 4.9 (11)		NS
30 Week	(VIRG)42.48 ± 13.04 (14)	37.33 ± 4.2 (5)		NS

NS=Not significant (p>0.05)

Sampling was done in non-fasted mice. These data in Table 2 show no significant differences in alkaline phosphatase between 8 and 26 weeks of age. In addition, the serum skeletal alkaline phosphatase was not significantly different in the B6C3HF1s as compared with the two parental strains. In the histomorphometry data described below, we find that the higher bone density in the C3H versus the B6 is already apparent by 8 weeks of age. Thus, it will be important in the future to sample for these biochemical markers of bone formation and resorption at earlier ages, such as 4 and 6 weeks.

2). Bone Resorption Markers

Osteoclastic Acid Phosphatase. Acid phosphatases are iron containing, cationic glycoproteins with molecular weights of around 35 kDa and a monomeric peptide structure. Osteoclastic tartrate resistant acid phosphatase (sTRAP) is one of the several isoenzymes (type 0-5) of acid phosphatase that can be identified by gel electrophoresis. Apart from the bone, tartrate resistant acid phosphatase has also been isolated from other tissues, including spleen, lung, placenta, macrophages and blood erythrocytes. Serum type 5 TRAP activity is normally expressed by osteoclasts, alveolar and monocyte-derived macrophages, and the placenta. Serum concentration of osteoclastic TRAP has been shown to correlate with indices of bone resorption.

Osteoclastic tartrate resistant acid phosphatase can be measured in serum by calorimetric methods using p-nitrophenyl phosphate as substrate. However, this method is not specific and results may be misleading in the presence of increased concentration of these interfering isoenzymes. Therefore, we have developed a radioimmunoassay which will be utilized to measure osteoclastic acid phosphatase in mouse serum.

The antibodies used in the development of this radioimmunoassay have been generated in goats using a linear synthetic peptide derived from the published sequence of mouse type-5 tartrate resistant acid phosphatase. A radioimmunoassay has been standardized using the goat antibody and synthetic peptide as tracer and calibrator. The RIA exhibited intra-assay (n=8) and inter-assay (n=3) variation between 8.07% and 16.03%. The assay is currently validated for the specificity of the antibodies against other tissue specific acid phosphatases (isolated from mouse spleen, erythrocytes, platelets, prostate, and osteoblasts).

As indicated above, we have completed the development of this assay and it will soon be ready to apply to our serum samples. However, before doing so, we plan to identify the circulating moiety of acid phosphatase that is identified by our antiserum to establish that the antibody does not cross-react with non-acid phosphatase proteins. Once these validation procedures are completed, it will then be applied to the samples that have already been collected for this study.

3) Other Assays

(a) Serum IGF-I. During the course of our work, we found that the serum IGF level was higher in the C3H mice than in the B6 mice at 8 weeks of age (720 ± 25 ng/ml vs. 460 ± 20 ng/ml, $p<.001$). Therefore, we developed an assay to measure serum growth hormone in order to determine whether an increase in growth hormone might be responsible for the increase in the serum IGF-I values in the C3H as opposed to the B6 mice.

(b) Mouse Growth Hormone (GH). The methodology that we developed to measure serum growth hormone follows. Mouse GH has been quantitated by a radioimmunoassay that was developed using antibodies generated in rhesus monkeys against a rat recombinant GH. Affinity purified mouse GH is used for making iodinated tracer and calibrator. Sensitivity of the GH RIA was 0.61 ng/ml. The RIA displays intra-assay (n=8) variation of 7.04% (CV). Averaged recovery from mouse

serum diluted up to 8 folds was 111.4% (CV). Averaged spiked recovery was 102% (when mouse GH, at concentration of 100 ng/ml, was added to three different mouse sera).

We found that, in the C3H mice compared to the B6 mice at 8 weeks of age, serum growth hormone was 10.2 ± 5 ng/ml vs. 4.4 ± 1 ng/ml, $p<.002$, indicating that growth hormone values in the C3H mice are more than double those in the B6 mice. Therefore, differences in GH secretion could be responsible for the increase in the serum IGF-I in the C3H as compared with the B6 mice.

(c) Creatinine. The methodology that we have utilized to make the creatinine measurements in these mice is described below.

Creatinine is measured by Jaffe reaction, where a yellow/orange color forms when creatinine is treated with alkaline picrate. Interferences from sample are eliminated by measuring the reaction before and after treatment with acid as the yellow color derived from creatinine is destroyed by acid treatment faster than the color derived from the interferences. The calorimetric assay shows intra-assay (n=20) and interassay (n=5) variation (CVs) between 1.01% and 4.6%.

Because the GH and IGF levels were higher in the C3H versus the B6 mice, we sought to test the hypothesis that muscle mass would be greater in the C3H versus the B6 mice. In order to get an estimate of muscle mass, we measured the 24-hour urine creatinine levels. We found that the 24-hour urine creatinine in C3H mice versus the B6 mice was 0.47 ± 0.15 ng/day vs. 0.32 ± 0.13 ng/day, $p<.02$. These data are consistent with the conclusion that there is a greater muscle mass, as well as a greater bone density, in the C3H versus the B6 mice.

b) Histomorphometric Measurements of Bone Formation and Bone Resorption at the periosteal and endosteal bone of the femoral diaphysis.

The methodology that we have developed to make bone histomorphometric measurements of the tibial diaphysis is described below. This methodology is similar to the methodology that we developed earlier for rat bones; however, it has not been applied in the past to mice.

1) Bone Histomorphometric Methods. The tibia was defleshed and stored in 70% ethanol at 4-5lC. Subsequently, each tibia was rinsed with d.i. water and sawed into one thin cross section about 50 mm in thickness at the fibular junction using a low speed saw (IsometΣ, Buehler Ltd., Lake Bluff, IL). The bone specimen was oriented at a position where the long axis of the diaphysis was perpendicular to the diamond saw. The cross-section was further ground to a thickness of about 30 mm, and mounted unstained in Fluoromount-G (Fisher Scientific, Pittsburgh, PA), a water-soluble, non-fluorescent mounting medium.

All bone measurements were evaluated with the OsteoMeasureΣ system with a digitizing tablet (OsteoMetrics, Inc., Atlanta, GA). Area and perimeter were measured at 2X microscope objective magnification. Measurements of the total tetracycline labeled surface (=dL+1/2sL) and width of tetracycline double labeled surface were under UV light and at 20X objective magnification. The mineral apposition rate (MAR, mm/day) was derived by dividing the mean of the widths of double labels by the interlabel time. The bone formation rate (BFR, mm2x10-3/day) was the product of the total label surface multiplied by MAR. The coefficient of variation by making three repeated measurements on five bone sections were as follows: cross-section area 0.78%, medullary area 1.86%, periosteal perimeter 0.57%, endosteal perimeter 1.36%, total label surface 6.44%, MAR 4.98%, and BFR 1.64%.

2) Histomorphometric Results

(a) Periosteal Bone Formation. **Table 3** provides our data on periosteal bone formation in C3H and B6 mice.

Table 3. Periosteal bone formation C3H and B6 mice at 8, 16, and 26 weeks of age (mean ± SE).

	cross-section area mm2	Label surface mm	MAR mm/day	BFR mm2x10-3/day
8 week-old				
C3H	0.79 ± 0.01 (n=9)	2.14 ± 0.11 (n=9)	2.74 ± 0.07 (n=9c	5.87 ± 0.34 (n=9)c
B6	0.82 ± 0.01 (n=9)	1.82 ± 0.20 (n=8)	1.68 ± 0.08 (n=8)	3.05 ± 0.35 (n=8)
12 week-old				
C3H	0.88 ± 0.01 (n=9)a	1.84 ± 0.04 (n=7)c	2.17 ± 0.13 (n=7)c	3.99 ± 0.24 (n=7)c
B6	0.83 ± 0.01 (n=10)	1.08 ± 0.12 (n=10)	0.93 ± 0.04 (n=10)	1.02 ± 0.14 (n=10)
16 week-old				
C3H	0.87 ± 0.01 (n=9)	1.81 ± 0.06 (n=7)b	0.81l0.03 (n=7)	1.46 ± 0.03 (n=7)
B6	0.86 ± 0.02 (n=9)	1.13 ± 0.19 (n=8)	0.92 ± 0.11 (n=7)	1.22 ± 0.30 (n=7)
26 week-old				
C3H	0.92 ± 0.02 (n=11)	1.63 ± 0.13 (n=10)	0.71 ± 0.03 (n=10)	1.17 ± 0.13 (n=10)
B6	0.88 ± 0.02 (n=9)	1.39 ± 0.05 (n=7)	0.70 ± 0.04 (n=7)	0.99 ± 0.09 (n=7)

a P<0.05; b P<0.01; cP<0.001

The data in **Table 3** show that there is no difference in the total cross-sectional area between the C3H and the B6 mice, which means that the external size of the bone is similar in these two inbred strains of mice. There was, however, a greater mineral apposition rate in the C3H than the B6 mice at 8 weeks of age. This was significant at p<.001. Because of this, there was also a greater bone formation rate in mm2 x 10-3 per day, which was significant at p<.001. The greater bone formation rate in the C3H versus the B6 mice was also seen at 12 weeks of age. However, at 16 and, also, 26 weeks of age, there was no significant difference in the periosteal bone formation rates between these two strains of mice. Interestingly, at 26 weeks of age the bone size, as measured by the total cross-sectional area, was not significantly different between these two strains of mice. At 8 weeks, the cross-sectional area was slightly less in the C3H than the B6, whereas by 26 weeks the cross-sectional area was slightly more in the C3H than the B6, but these two differences were not statistically significant. Nonetheless, these cross-sectional area data are consistent with the greater bone formation rate in the C3H than the B6.

(b) Endosteal Bone Formation. The data on endosteal bone formation in **Table 4** below indicate that, at 8 weeks of age, the medullary cavity area is lower in the C3H than the B6 mice. In addition, the endosteal bone formation rate was greater in the C3H than the B6 mice, though the differences were not statistically significant. The same pattern was seen in the 12-week old mice, with the exception that at that time point there was a significantly greater bone formation rate at the endosteum in the C3H versus the B6 mice, p<.01. At 16 weeks and 26 weeks, the smaller medullary cavity in the C3H versus the B6 mice was maintained. However, by 26 weeks of age, instead of the

C3H mice having a greater endosteal bone formation rate, the endosteal bone formation rate was significantly greater in the B6 mice than the C3H mice ($p<.001$). These data show that, as the mice age, there is a consistently smaller medullary cavity in the C3H than the B6 mice. This is associated with a greater bone formation rate in the C3H than the B6 mice through the 16-week age point. Since over this age-range the difference in medullary cavity area did not change, it follows that bone resorption also must have been greater in the C3H than the B6 mice during this age-interval between 8 and 16 weeks of age.

Table 4. Endosteal bone formation rates in C3H versus B6 mice (mean ± SE).

Age	Medullary area mm2	Label surface mm	MAR mm/day	BFR mm2x10-3/day
8 week-old				
C3H	0.19 ± 0.00 (n=9)c	1.23 ± 0.04 (n=9)c	2.47 ± 0.14 (n=9)c	3.08 ± 0.26 (n=9)
B6	0.33 ± 0.01 (n=9)	1.78 ± 0.04 (n=9)	1.60 ± 0.05 (n=9)	2.85 ± 0.10 (n=9)
12 week-old				
C3H	0.15 ± 0.00 (n=9)c	1.14 ± 0.04 (n=9)a	2.15 ± 0.11 (n=9)c	2.45 ± 0.1 1 (n=9)b
B6	0.30 ± 0.02 (n=10)	1.36 ± 0.08 (n=10)	1.11 ± 0.04 (n=10)	1.48 ± 0.06 (n=10)
16 week-old				
C3H	0.14 ± 0.01 (n=9)c	1.21 ± 0.07 (n=7)a	1.14 ± 0.04 (n=7)a	1.39 ± 0.11 (n=7)
B6	0.31 ± 0.01 (n=9)	1.50 ± 0.08 (n=9)	0.97 ± 0.06 (n=9)	1.49 ± 0.15 (n=9)
26 week-old				
C3H	0.14 ± 0.00 (n=11)c	0.70 ± 0.04 (n=8)c	0.88 ± 0.10 (n=8)	0.62 ± 0.09 (n=8)c
B6	0.27 ± 0.01 (n=9)	1.56 ± 0.06 (n=8)	1.15 ± 0.07 (n=8)	1.79 ± 0.12 (n=8)

a $P<0.05$; b$P<0.01$; c$P<0.001$

In as much as the medullary cavity area difference at 8 weeks of age is as much as that seen at 26 weeks of age, it follows that the difference in peak bone density between the two strains of animals, which is inversely proportional to medullary cavity area, is achieved prior to the 8-week sampling point. This means that, in order to know the reason for the increase in peak bone density in terms of bone formation and bone resorption at the endosteum, we need to measure time points earlier than 8 weeks of age.

We are now in the process of validating our bone resorption measurements and they will be presented in the next progress report.

CONCLUSIONS

Specific Aim 1. The QTL mapping strategy is work very well and is demonstrating that BMD has a substantial genetic basis. These preliminary mapping data suggest that at least 6 loci account for the difference in bone density between B6 and C3H mice, and at least 8 loci account for differences between B6 and CAST/EiJ. Loci on Chr 1, 5, and 18 may be the same in both crosses. Thus, we have 10 loci underlying bone density among these 3 inbred strains, with loci dividing into those with major effects and those with minor effects on BMD. We will not begin a serious search for candidate genes until the initial mapping effort is completed. We anticipate additional F2 progeny to reveal more loci in the B6C3F2 progeny and improve the statistical assessment of variance accounted for in this cross. The B6CASTF2 cross served its purposes fully and will not be pursued further at this time.

Biological stimulus tests show that genetic differences between C3H and B6 resulting in high or low bone density, respectively, are not regulated by either ovarian steroid or mechanical tension placed on bone by muscle action. In contrast, the GH/IGF-I axis merits additional experimental analyses for its role in high bone density. Clearly, several factors within the paradigm of bone formation and bone resorption contribute to high bone density in C3H mice. Determining what these mechanism are will be of very real interest to scientists interested in pediatric, geriatric, and space medicine.

Specific Aim 2. The most important finding of the study, thus far, is that we have unambiguous data to indicate that the essential physiological events leading to differences in peak BMD between the two strains of mice has already occurred prior to our 8-week sampling time. In other words, the peak BMD difference was essentially stable after the 8-week sampling time. This means that earlier time points must be sampled to determine when the peak bone density occurs. More importantly, in order to know the skeletal formation and resorption dynamics that account for the difference in peak bone density between the C3H and B6, we must make our dynamic measurements during the time when peak bone density is occurring, namely before 8 weeks of age.

Another finding that is apparent from our results is that the differences in bone formation and bone resorption are not necessarily constant at the endosteum throughout the longitudinal study. This would suggest that there are genes that determine peak bone density and then additional genes, or a different regulation of the same genes, that control subsequent growth and development. Consequently, in order to determine those genes that are responsible for peak bone density, we must sample the animals at relatively early time points.

The discovery of an increase in serum IGF-I in the C3H over the B6 mice could be an important phenotypic difference, because the differential serum IGF-I could contribute to the differential peak bone density between these two inbred strains of mice. Furthermore, the finding that GH was correspondingly higher in the C3H mice versus the B6 mice points in the direction of gGH regulation as a possible cause of the IGF-I difference between the two strains of mice. Consistent with the idea that growth hormone and IGF-I are up-regulated in the C3H versus the B6 mice is the finding that the 24-hour urine creatinine was higher in the C3H than the B6 mice, indicating a greater muscle mass. This indirect finding of a greater muscle mass needs to be taken into consideration when interpreting our bone dynamic data because muscle mass, per se, could have an influence on bone remodeling and modeling dynamics.

Finally, it will be important to carefully examine trabecular bone to make certain that the changes in trabecular bone are behaving similar to those of endosteal cortical bone. If not, it could mean that there are genes regulating peak bone density that are different in trabecular bone than in cortical endosteal bone.

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